Mitogenic Activity of Zeranol in Human Breast Cancer Cells Is Enhanced by Leptin and Suppressed by Gossypol

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Abstract. *Background: The molecular links between breast cancer and obesity have been studied for many years. Obesity significantly increases the incidence rate and chance of morbidity of breast cancer. Leptin, mainly secreted by adipocytes, plays an important role in breast cancer development. Leptin expression is up-regulated in obesity and it can promote breast cancer cell growth. Zeranol is used as an anabolic growth promoter to stimulate cattle growth in the U.S. beef industry. (–)-Gossypol, a natural polyphenolic compound extracted from cottonseed, is an anticancer chemopreventive agent. Materials and Methods: Zeranol, leptin and (–)-gossypol were used to investigate MCF-7 Adr cell growth. Results: Leptin enhanced the sensitivity of MCF-7 Adr cells to zeranol and increased cell growth. Exposure to zeranol may lead to initiation of transformation of normal breast cells to breast pre-neoplastic cells. Conclusion: It is suggested that obese individuals may be at greater risk of developing zeranol-induced breast cancer.*

Breast cancer is a worldwide disease, causing over 40,000 women to die of each year in the U.S. (1). One of the currently known risk factors of breast cancer is obesity, which has become a major public health concern (1). The incidence of breast cancer is increased with obesity, and morbidity is also increased in obese cancer patients as compared to cancer patients with normal or low weight (2). The relationship between breast cancer and obesity has been studied for more than 40 years (3).

Leptin, a transcriptional product of the *ob* gene, plays an important role in breast cancer development and has been studied since its discovery in 1994 (4). It is a 167-amino acid protein with a molecular mass of 16 kDa. Besides its involvement in appetite regulation and energy balance by sending signals to the hypothalamus (5), leptin has a number of other regulatory functions such as ensuring normal mammary gland development, bone development, fetal development, sex maturation, angiogenesis, lactation, hematopoiesis, and immune responses (reviewed in 1, 2, 6). Additionally, leptin is necessary for normal mammary gland development in rodents (2). Animals and humans with defective leptin or with mutated leptin receptor genes are obese (5, 7). The serum level of leptin in obese cancer patients was determined to be significantly higher than that in a healthy control group. In clinical studies, the serum leptin level in prostate cancer patients was found to be higher than that in a healthy control group, and was correlated with prostate-specific antigen (8, 9). In a breast cancer research program, 60% (20/35) of the patients expressed leptin, while none out of four cases with normal breast tissue expressed leptin (10).

Leptin has a direct mitogenic effect on human breast cancer cells (11); therefore, the inhibition of leptin may contribute to the prevention and treatment of breast cancer (12). Leptin expression is up-regulated in obesity (2) and it can promote breast cancer cell growth by directly affecting the estrogen receptor (ER) pathway (1, 2). The leptin receptor (ObR), localized in the cell membrane of various types of tissues, including breast cancer (13), has been identified as having one long isoform and five short isoforms (1). Both short and long isoforms are expressed in normal mammary epithelial cells during pregnancy and lactation (1, 2, 14). Such expression suggests an autocrine action of leptin in the regulation of mammary gland growth and development. Furthermore, because it is commonly expressed in breast cancer, the gene could serve as a tumor marker, or a prognostic or diagnostic factor (1, 10, 14).

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Like other growth factors and cytokines, leptin and its receptor are present in human serum, and both play a role in human cancer development (15). Because leptin was found to be associated with several types of cancer (2, 15), researchers have attempted to find the relationship and mechanisms of leptin action in several prostate cancer (9), gastric cancer (16), esophageal adenocarcinoma (17), hepatocellular carcinoma (18), gallbladder cancer (19), cholangiocarcinoma (20), and breast cancer (21-27). Ishikawa *et al.* found that leptin was overexpressed in breast cancer cells (13) and likewise concluded that high leptin levels in obese breast cancer patients might play a role in the development of antiestrogen resistance (26). Leptin is not expressed in normal breast tissue but exists near malignant breast lesions (11), while its receptors were detectable in cancer cells but undetectable in normal mammary epithelial cells (13). In addition to its mitogenic effects, leptin can promote T47-D cell line proliferation (14) and a high level of leptin might contribute to the development of a more aggressive malignant phenotype (28). ICI 182,780 is a pure estrogen antagonist approved for treatment of breast cancer patients who failed to respond to tamoxifen therapy. The power of leptin to stimulate human MCF-7 cell growth and to counteract the effects of ICI 182,780 strongly suggests that leptin acts as a paracrine/endocrine growth factor towards mammary epithelial cells (26, 27). Chen *et al.* also found that leptin increases ZR-75-1 breast cell growth by up-regulating cyclin D1 and down-regulating P53 (29). Because it stimulates estrogen biosynthesis through induction of aromatase activity and modulation of ER α activity, leptin has been characterized as a growth factor for breast cancer. High levels of leptin in obese breast cancer patients might play a remarkable role in breast cancer cell proliferation, invasion, and metastasis (2, 16, 30)

Estrogen has been regarded as a positive regulator of leptin production (31), and the leptin level in breast cancer patients treated with tamoxifen is significantly higher than levels in the control group (32). Thorn *et al.* found that as another risk of breast cancer development, estrogen can modulate ObR expression in some estrogen-responsive tissues (31, 33). Zearalenone which is a stable natural product that with mimics estrogen activity is a carcinogen and thus hazardous to human health (34). Zeranol, produced from zearalenone, is a non-estrogenic anabolic growth promoter and is used to stimulate cattle growth in the U. S. beef industry (35). Both zearalenone and zeranol can bind to the active site of human ER α and ER β in a similar manner to 17 β-estradiol (36). As food contaminant, the intake of zeranol is very hard to avoid (34). Researchers also found that zeranol did not change the serum leptin level in growing wethers (37). At low concentration, it can increase ERα-positive cell growth, but a high concentration of zeranol can reduce growth of both ERα-positive and -negative cell lines

(38). Moreover, our previous data showed that zeranol was able to transform human normal breast epithelial cell and increase human breast cell growth in a dose-dependent manner (35) and can down-regulate estrogen-regulated human breast cancer candidate suppressor gene, protein tyrosine phosphatase γ (PTPγ) expression (39). We also have previous data showing that leptin induces human breast cancer epithelial cell sensitivity to zeranol (data in preparation for publication).

Another natural polyphenolic compound extracted from cottonseed and used as an anticancer chemopreventive agent, gossypol, can inhibit various types of cancer cell growth such as colon (40), prostate (41), and breast cancer (42). It is suggested gossypol could be used as a potential chemopreventive food component. Our laboratory also demonstrated that gossypol has anticancer activity against multidrug resistant human breast cancer cells (43), with (–)-gossypol having the strongest effect among the three isoforms (data not shown). The objective of this research was to investigate the mechanisms of the suppression of zeranol- and leptin-induced proliferation of MCF-7 Adr cells by using (–)-gossypol as the main chemopreventive agent

Materials and Methods

Reagents. Recombinant human leptin was ordered from the R&D Systems (Minneapolis, MN, USA) and was prepared as stock solution of 1 mg/ml in sterile 20 mM Tris/HCl (pH 8.0) at –20˚C; (–)-gossypol was provided by USDA Southern Regional Research Center (New Orleans, LA, USA), and was prepared as 50% stock solution in dimethyl sulfoxide (DMSO); zeranol was purchased from Sigma Chemical Company (St. Louis, MO, USA).

Cell culture. MCF-7 Adr cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in phenol red-free high calcium Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) (DMEM/F12) (1.05 mM CaCl$_2$) containing 5% fetal bovine serum (FBS) and antibiotic-antimycotic (100 unit/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B) (GibcoBRL, Bethesda, MD, USA) in a 75 cm^2 culture flask in a humidified incubator (5% CO$_2$, 95% air, 37˚C). When the cells grew to 85-90% confluence, cells were subcultured into 75 cm^2 culture flasks at a ratio of 1 flask to 5 flasks as described above. Cells were dissociated using 1 ml of 0.5% trypsin-5.3 mM EDTA (GibcoBRL) in PBS for 3 minutes at 37˚C. The trypsinization was stopped by the addition of 10 ml of culture medium with 10% FBS. After centrifugation, the dissociated cells were resuspended in the culture medium with 10% FBS and subcultured into 75 cm^2 culture flasks at a ratio of 1 flask to 5 flasks.

Cell proliferation assay (MTT assay). One hundred μl MCF-7 cells 3,000/well were placed into 96-well plates in DMEM/F12 medium and incubated in 37˚C for 24 hours. After this time, the medium was changed by 100 μl phenol red-free high calcium DMEM/F12 supplemented with 0.2% BSA, and the plate was incubated at 37˚C for another 24 hours. Treatment of 1, 3, 6, or 12 nM leptin or 10, 20, or 30 nM zeranol was given, 0.1% DMSO being given to the

control group. Twenty-four hours later, cell growth was measured. The cell proliferation was measured by adding 20 µl of a fresh mixture of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) (20:1) solution (Promega, Madison, WI, USA) to each well. After incubation at 37˚C for 3-6 hours, OD values were measured by kinetic microplate reader (Molecular Devices Cooperation, Menlo Park, CA, USA) at 490 nm wavelength and cell growth was compared.

In the MTT assay of combination of leptin with zeranol and (–)-gossypol, the concentration used was 6 nM, 30 nM, and 5.0 µM for leptin, zeranol, and (–)-gossypol respectively, alone or combined; 0.1% DMSO was given to the control group. After 24-hour treatment, cell growth was measured as described above.

In the experiment investigating leptin-induced sensitivity to zeranol, treatment was given to MCF-7 Adr cells in five groups: i) pre-treatment with leptin group (pre-lp): cells were treated with 6 nM leptin for 24 hours then treated with 10, 20, or 30 nM zeranol for 24 hours; ii) post-treatment with leptin group (post-lp): cells were treated with 10, 20, or 30 nM zeranol for 24 hours then 6 nM leptin for the following 24 hours; iii) leptin group (lp): cells were only treated with 6 nM leptin for 48 hours; iv) zeranol only group (Z): cells were only treated with 10, 20, or 30 nM zeranol; v) control group (CT): cells were only treated with 0.1% DMSO. All media were changed every 24 hours. After 48 hours treatment, cell growth was measured as described above.

Cell treatment for RNA and PCR analyses. A total of 10^5 viable cells/well MCF-7 Adr cells were seeded in 6-well plates in 5 ml phenol red-free high calcium DMEM/F12 medium. Twenty four hours later, the medium was replaced with phenol red-free high calcium DMEM/F12 supplemented with 5% dextran-coated charcoal (DCC), or the cells were cultured overnight. After 24 hours, 6 nM leptin, 10, 20, or 30 nM zeranol alone or combined with 6 nM leptin, or the combination of 6 nM leptin with 30 nM zeranol and 5 µM (–)-gossypol was given to MCF-7 Adr cells which were then incubated for a further 24 hours.

RNA isolation and cDNA synthesis. After cells were treated for 24 hours, total RNA was isolated in 1 ml TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration was measured by DU-70 spectrophotometer (Beckman Instruments Inc. Fullerton, CA, USA). RNA (1 µg) from cultured cells was reverse transcribed with 200 U M-MLV Reverse Transcriptase (Invitrogen) at 37˚C for 50 minutes and then 70˚C for 15 minutes in the presence of 1 µl 10 mM dNTP (10 mM each dATP, dGTP, dCTP, and dTTP at neutral PH) (Invitrogen), 1 µl 50 µM Random hexamer (Amersham, Piscataway, NJ, USA), RNase Inhibitor (Invitrogen), 10 µl 5x First Strand buffer, 5 µl 0.1M DTT and 1µl RNase Inhibitor (Invitrogen) in a total volume of 50 µl in a gradient mastercycle (Eppendorf®, Westbury, NY, USA).

Reverse transcription-polymerase chain reaction. RT-PCR conditions were optimized for every primer and performed with a thermocycler Gene Amp PCR (Eppendorf®). A volume of 2 µl of the newly synthesized cDNA was used as templates for RT-PCR. PCR conditions were optimized for $MgCl_2$ concentration, annealing temperature, and cycle number for the amplification of each of the PCR products (cyclin D1, P53, or ObR). Under optimal conditions, 1 U platinum Taq DNA polymerase (GibcoBRL) was added in a total volume of 25 µl.

Primers for cyclin D1: Upper primer: 5'-GCT CCT GTG CTG CGA AGT GG-3', lower primer: 5'-TGG AGG CGT CGG TGT AGA TG-3' (product size 372 bp), at 95˚C for 5 min, 27 cycles of 94˚C for 45 s, 54˚C for 45 s, 72˚C for 60 s, then extension at 72˚C for 10 min. Primers for P53: Upper primer: 5'-CAT GAC GGA GGT TGT GAG GC-3', lower primer: 5'-CGC AAA TTT CCT TCC ACT CG-3' (product size 336 bp), at 95˚C for 5 min, 31 cycles of 95˚C for 45 s, 57˚C for 45 s, and 72˚C for 45 s, then extension at 72˚C for 10 min. Primers for ObR common domain: Upper primer: 5'-CAT TTT ATC CCC ATT GAG AAG TA-3', lower primer: 5'-CTG AAA ATT AAG TCC TTG TGC CCA G-3' (product size 273 bp), at 95˚C for 5 min, 30 cycles of 95˚C for 40 s, 60˚C for 50 s, 72˚C for 50 s, then extension at 72˚C for 10 min. Primers for 36B4: Upper primer 5'-AAA CTG CTG CCT CAT ATC CG-3', lower primer 5'- TTT CAG CAA GTG GGA AGG TG-3' (product size 563 bp), at 95˚C for 5 min, 24 cycles of 95˚C for 60 s, 63˚C for 60 s, 72˚C for 60 s, then extension at 72˚C for 10 min. Pure H_2O was used as negative control in order to detect genomic DNA contamination and 36 B4 as internal control whose RNA is unmodified by treatment.

The final RT-PCR products (10 µl) mixed with 1 µl 10 x loading buffer were separated on 1.5% agarose gel and visualized by staining with ethidium bromide. Electronic images were taken by a FUJIFILM LAS-3000 image system (FUJIFILM Medical Systems USA, Inc. Stanford, CT, USA). The densities of specific bands were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The results were presented as the ratio of cyclin D1 to 36B4, p53 to 36 B4, and ObR to 36 B4.

Statistical analysis. The results for the cell proliferation assay are presented as mean±standard deviation (SD) for 4 replicate culture wells. Analysis was performed by using Minitab 15 (Minitab Inc. PA, USA). Statistical difference was determined by using two-sample *t*-test analyses for independent samples. *P*-values of less than 0.05 were considered statistically significant.

Results

Leptin (6 nM) and zeranol (20 nM) increased MCF-7 Adr cell proliferation and 6 nM leptin induced MCF-7 Adr cell sensitivity to zeranol. As shown in Figure 1A, 20 and 30 nM zeranol significantly increased MCF-7 Adr cell growth by 26% and 35% respectively. Figure 1B shows 6 and 12 nM leptin significantly increased MCF-7 Adr cell growth by at least 120% and in a dose-dependent manner. Figure 2A shows that cells pre-treated with leptin grew significantly faster than post-treated cells and the non-leptin group. The group treated with 6 nM leptin and 30 nM zeranol exhibited the most cell proliferation compared to the 10 nM and 20 nM zeranol groups. The combination of 6 nM leptin with 30 nM zeranol significantly promoted cell growth compared to 6 nM leptin and 30 nM zeranol alone while (–)-gossypol at 5 µM significantly suppressed the stimulation induced by zeranol combined with leptin (Figure 3A).

Combination of leptin (6 nM) and zeranol (30 nM) increased cyclin D1 expression and reduced P53 expression in MCF-7 Adr cell. Figure 3B shows that 6 nM leptin and 30 nM
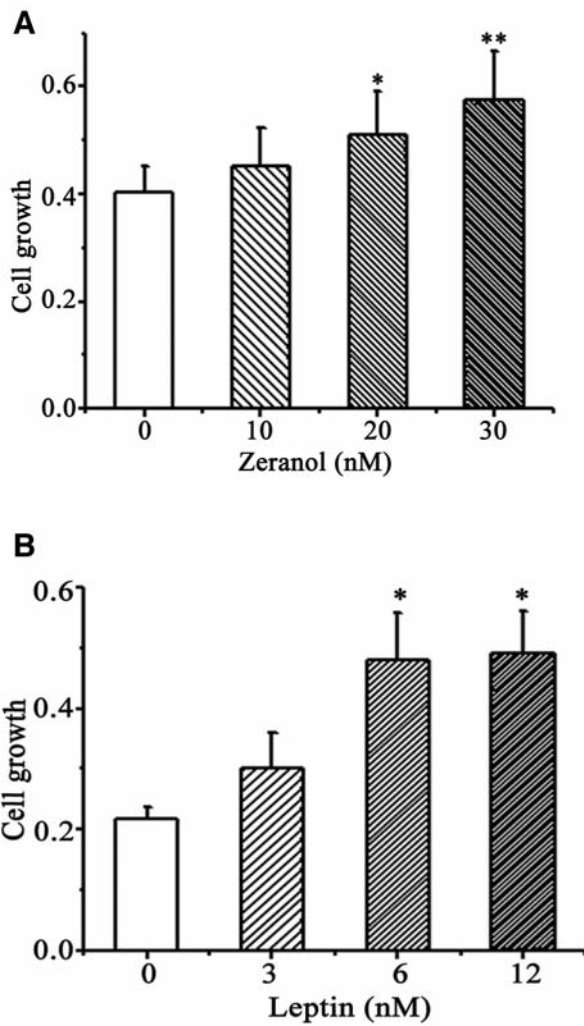
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Figure 1. *A, The effect of zeranol on MCF-7 Adr cell growth. MCF-7 Adr cells were treated with 10, 20 or 30 nM Zeranol; 0.1 % DMSO as control. Significant differences in MCF-7 Adr cell growth compared to the control group at *p<0.05, **p<0.01. B, The effect of leptin on MCF-7 Adr cell growth. MCF-7 Adr cells were treated with 3, 6, or 12 nM of leptin; 0.1% DMSO as control. Significant differences in MCF-7 Adr cell growth compared to the control group at *p<0.05.*
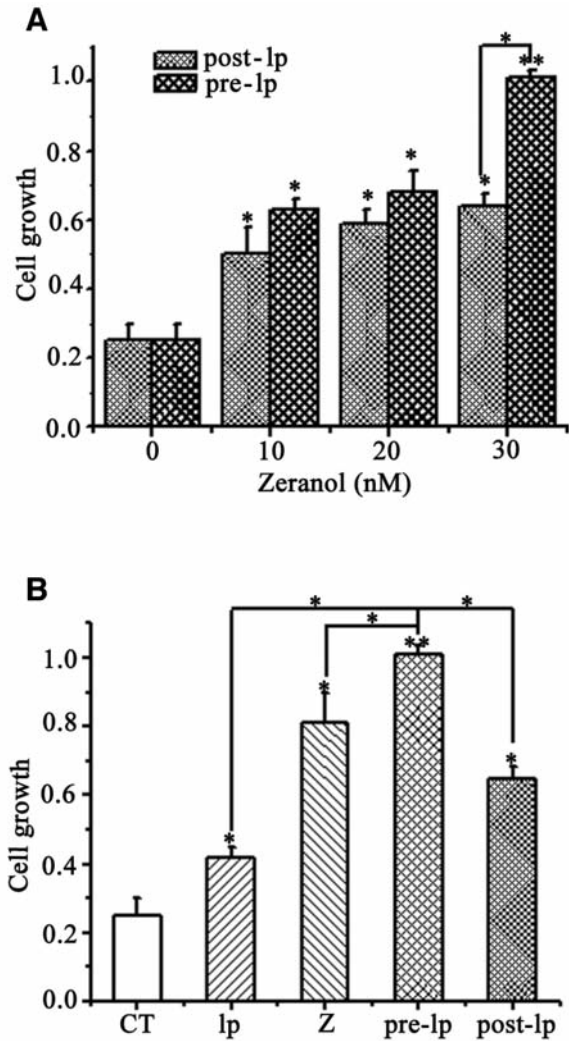
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Figure 2. *A, Comparison of MCF-7 Adr cell growth after pre-treatment with leptin (pre-lp) and post-treatment with leptin (post-lp). Significant differences in MCF-7 Adr cell growth compared to the control group and between pre-lp and post-lp groups at *p<0.05, **p<0.01. B, MCF-7 Adr cells were treated with 6 nM leptin (lp) or 30 nM Zeranol (Z) in the first and/or second 24 hours. Significant differences in MCF-7 Adr cell growth compared to the control group and between any two groups at *p<0.05, **p<0.01).*

zeranol alone increased *cyclin D1* mRNA expression without significant difference. When they were combined, *cyclin D1* mRNA expression significantly increased while (−)-gossypol at 5 μM counteracted the stimulatory effect.

In addition, 6 nM leptin and 30 nM zeranol reduced *P53* mRNA expression without significant difference as compared to the control group. When they were combined, the *P53* mRNA expression was significantly reduced when compared to the control group, while (−)-gossypol at 5 μM reversed their combination effect (Figure 3C). There was a significant difference between the combination of leptin and zeranol with and without (−)-gossypol.

Zeranol (30 nM) significantly increased ObR expression in MCF-7 Adr cells. As shown in Figure 4, comparing to the control group, 30 nM zeranol can significantly increased ObR expression in MCF-7 Adr cells by about 40%, while 10 and 20 nM zeranol increased the ObR expression but not significantly.

Discussion

Cyclin D1 is a cell cycle regulator and plays an important role in cell growth. The cyclin-dependent kinases (CDKs) cannot regulate cell growth without the cyclin subunit. By binding to
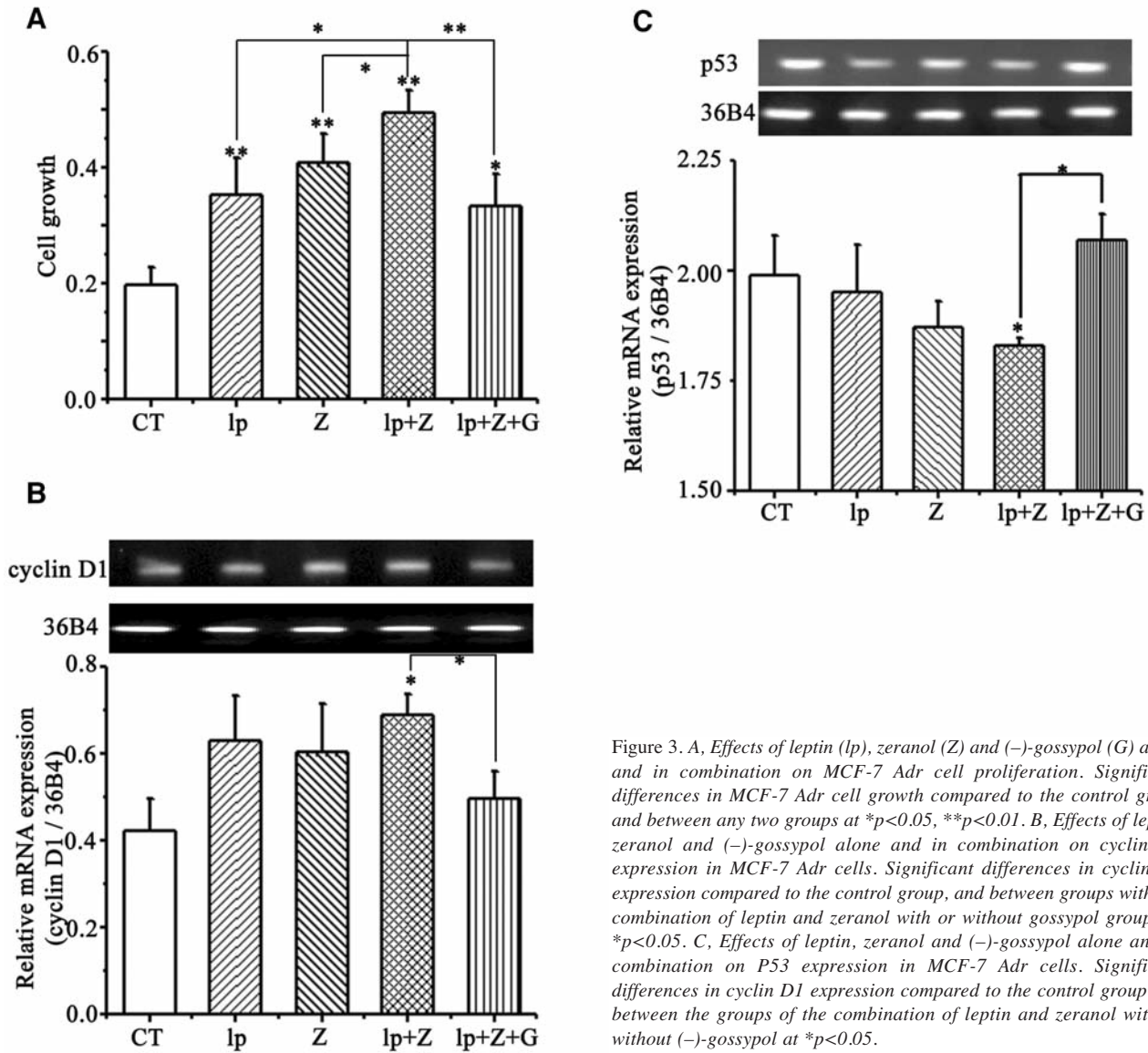
Figure 3. *A, Effects of leptin (lp), zeranol (Z) and (–)-gossypol (G) alone and in combination on MCF-7 Adr cell proliferation. Significant differences in MCF-7 Adr cell growth compared to the control group and between any two groups at *p<0.05, **p<0.01. B, Effects of leptin, zeranol and (–)-gossypol alone and in combination on cyclin D1 expression in MCF-7 Adr cells. Significant differences in cyclin D1 expression compared to the control group, and between groups with the combination of leptin and zeranol with or without gossypol groups at *p<0.05. C, Effects of leptin, zeranol and (–)-gossypol alone and in combination on P53 expression in MCF-7 Adr cells. Significant differences in cyclin D1 expression compared to the control group and between the groups of the combination of leptin and zeranol with or without (–)-gossypol at *p<0.05.*

cyclin D, cyclinD-CDK 4/6 constitutes the engine of the cell cycle machinery and affects the G_1-phase in cell growth. The cyclin D1 level can be modulated by changing growth factors in the medium used to culture cells. Leptin stimulates breast cancer cell growth by up-regulating *cyclin D1* expression. Moreover, Garofalo *et al*. found leptin could modulate both estrogen synthesis and estrogen receptor (ER) α activity (14, 15). Besides controlling the cell cycle, cyclin D1 was found to be associated with ER (44). Cyclin D1 can bind to the ER and stimulate its transcriptional activities. The cyclin D1 and ER complex may play a role in stimulating the tumor cell proliferation. On the other hand, *P53* is a tumor suppressor gene. It is reported that cyclin D1 expression can be regulated by P53 (44).

Our data show that 6 nM leptin or 30 nM zeranol alone had no affect on the *cyclin D1* expression but their combination significantly increased the expression of *cyclin D1* comparing to the control group. In our cell proliferation assay, cells pre-treated with leptin increased their sensitivity to zeranol. This can partly be explained by the fact that the combination resulted in high expression of cyclin D1. It is possible that if obese healthy women or breast cancer patients have higher leptin in their serum, the sensitivity of normal or cancerous breast cells may be increased by zeranol contained in beef. Under such circumstances, the risk of breast cancer may be increased by zeranol contained in beef. The risk of breast cancer may be increased because the
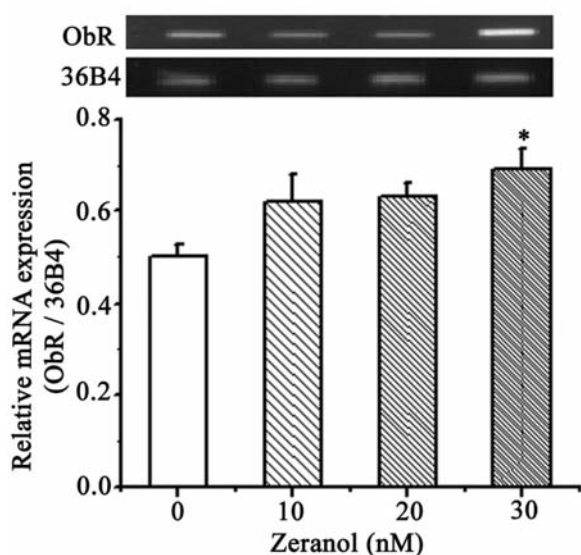
Figure 4. *Effects of zeranol on ObR expression in MCF-7 Adr cells. Significant difference in ObR expression compared to the control group at *p<0.05.*

breast cancer patients expressed more leptin than the control group (10). Moreover, we found that primary cultured human normal breast epithelial cells were more sensitive to leptin and zeranol than are MCF-7 Adr cells (data in preparation for publication).

According to other researchers, the serum level of leptin in breast cancer patients is higher than that in controls (32). Figure 3A shows that 30 nM zeranol stimulated cell growth more than 6 nM leptin. However, cells pre-treated with 6 nM leptin grew more than those only treated with 30 nM zeranol for 48 hours (Figure 2B). The pre-treatment of 6 nM leptin significantly stimulated cell growth, comparing to the other groups. This result supports our hypothesis that pre-treatment of 6 nM leptin for 24 hours can increase the sensitivity of MCF-7 Adr cell to zeranol and thus enhance the proliferation of MCF-7 Adr cells. This suggests a possible relationship between obesity and breast cancer and points to the potential risk for breast cancer in obese people, especially in those consuming meat from animals implanted with zeranol because leptin can increase the cell sensitivity to zeranol.

On the other hand, like estrogen modulating the ObR expression in some estrogen-responsive tissues (31), 30 nM zeranol can increase ObR expression in MCF-7 Adr cells to strengthen the action of leptin. The molecular action of leptin was initiated by leptin binding to leptin receptor ObR. When treated with zeranol, the MCF-7 Adr cells increased their expression of ObR and thus the action of leptin was amplified. This result indicates that zeranol might be more

harmful to obese people than normal weight people in increasing breast cancer risk (38) because their consumption of zeranol-containing products may amplify their chances of having breast cancer. On the other hand, (–)-gossypol can reverse the effect of the combination of leptin with zeranol on cell growth; it could be used in the treatment of breast cancer, especially in obese multidrug-resistant patients.

In summary, leptin appears to increase MCF-7 Adr cell growth *via* increasing *cyclin D1* mRNA expression. Leptin improves MCF-7 Adr cell sensitivity to zeranol and zeranol can strengthen the effect of leptin by increasing ObR expression in MCF-7 Adr cells. (–)-Gossypol can counteract the growth of breast cancer cells induced by leptin alone or combined with zeranol by down-regulating *cyclin D1*, and up-regulating *P53* mRNA expression. More mechanisms will be further studied in the future. Our report is the first to reveal that (–)-gossypol as a food component in cottonseed products may serve as a potential chemopreventive agent to suppress the stimulatory effect of zeranol and leptin on human breast cancer cells.

Acknowledgements

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